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Conserved Regulatory Mechanisms Controlling Aflatoxin and Sterigmatocystin Biosynthesis

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1. Introduction

Filamentous fungi produce a wide array of compounds known as secondary metabolites or natural products (i.e. Adrio and Demain, 2003; Reverberi et al 2010; Brakhage and Schroeckh 2011). The exact function of these compounds is unknown but it is postulated that they provide fungi with an advantage in their ecological niche (Keller et al., 2005; Georgianna and Payne 2009). The broad group of natural products includes compounds such as antibiotics, pigments and also mycotoxins (Fig.1). Among mycotoxins, aflatoxins (AF), specifically AF B1, are the most potent carcinogenic natural compounds known, and they are mainly produced by the opportunistic plant pathogens *Aspergillus flavus* and *Aspergillus parasiticus* (Squire 1989; Sweeny and Dobson 1999; Payne and Brown, 1998). Other AF-producers among *Aspergilli* include *Aspergillus nomius*, *Aspergillus ochraceoroseus*, *Aspergillus bombycis* and *Aspergillus pseudotamari* (Cary and Ehrlich, 2006). *Aspergillus flavus* and *Aspergillus parasiticus* have the ability to colonize oil seed crops of agricultural importance, such as corn, cotton, peanuts, sorghum and tree nuts. Ingestion of aflatoxin contaminated food can cause hepatocellular carcinoma, immunotoxicity, and teratogenic effects (Dvorackova and Kusak, 1990; Trail et al., 1995; Wogan et al., 1992). Various developed countries have strict regulation on the amount of AF allowed in food commodities. Contaminated crops above the permitted limit have to be destroyed, resulting in economic losses. The annual estimated loss due to AF contamination is attributed at approximately $270 million in the USA alone (Richard and Payne, 2003).

It is known that AF and a related mycotoxin called sterigmatocystin (ST) (Fig.2) are synthesized through the same conserved biosynthetic pathway, in which ST is the penultimate precursor. ST is produced by several *Aspergillus* species, including *Aspergillus nidulans*, one of the most characterized eukaryotic systems that has been used as a model organism for more than 60 years (Pontecorvo et al., 1953). The elevated number of characterized *A. nidulans* genes and mutant strains makes this model fungus ideal for genetic and molecular studies. There is a physical and genetic map of the eight chromosomes in *A. nidulans.* The whole *A. nidulans* genome has been sequenced and annotated, and has been compared with other *Aspergillus* genomes (David et al., 2008; Galagan et al., 2005). Structural and signaling pathway genes controlling ST production in *A. nidulans* are also found in AF-producer *Aspergillus* spp. (Hicks et al., 1997; reviewed by

Calvo et al., 2002; Calvo, 2008). For these reasons this model system is especially productive in the study of the AF/ST gene clusters and regulatory pathways directing mycotoxin production. In this review we will focus on these common regulatory mechanisms governing AF and ST biosynthesis in *A. flavus* and *A. parasiticus,* and in *A. nidulans*.

2. AF/ST gene clusters

ST/AF gene clusters remain some of the best characterized mycotoxin gene clusters. Both encode enzymes participating in 29 metabolic steps along with two regulatory proteins, AflR and AflS. (Cary et al., 2009). Both gene clusters extend to approximately 70 kb in the genome (Brown et al., 1996; Cary and Ehrlich, 2006; Ehrlich et al., 2005; Cary et al., 2009). The order and direction of genes in AF and ST clusters is conserved for all genes except four in AF pathway and three in ST which are inverted. It has been hypothesized that these differences might have been the result of gene reorganization from an ST-producing ancestor by recombination and duplications of near-telomeric regions where these clusters are found (Cary et al., 2009; Carbone et al., 2007). Based on phylogenetic analysis, the clusters may be 450 million years old (Galagan et al., 2005).

The enzymatic reactions and chemistry involved in the AF/ST biosynthetic pathway have already been extensively covered in previous reviews (Bhatnagar et al., 2003; Bennett and Klich, 2003; Hicks et al., 2002; Huffman et al., 2010; Keller et al., 2005; Minto and Townsend, 1997; Payne and Brown, 1998; Yabe and Nakajima, 2004), and will not be addressed in this review. Beyond the genus *Aspergillus*, other fungi present semi-conserved pathways, for example *Dothistroma septosporum*, which produces a metabolite that resembles versicolorin B, a precursor of both ST and AF. This suggests further cluster conservation across fungal genera (Bradshaw and Zhang, 2006).

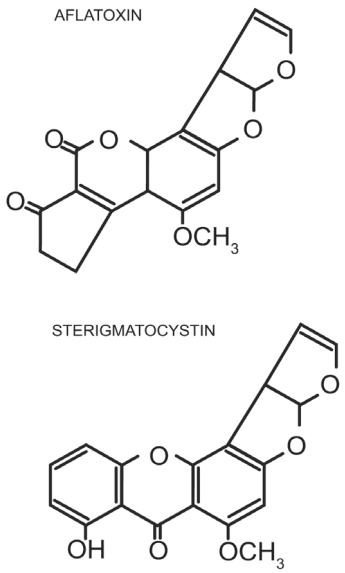

Fig. 1. Chemical structures of the mycotoxins aflatoxin and sterigmatocystin.

3. AflR and AflS

In addition to the structural genes, two regulatory genes are also included in these clusters, *aflR* and *aflS* (formerly known as *aflJ)*. The gene products AflR and AflS, are specific regulators for AF/ST gene cluster activation and for concomitant AF/ST production. AflR, encodes a transcription factor with a cysteine rich Gal4 type bi-nuclear zinc finger cluster (Chang et al., 1995; Yu et al., 1996). AflR has been shown to control most of the steps of the ST/AF biosynthetic pathway. AflR binds DNA at the consensus motif 5'TCGN5CGA3' in the promoter of biosynthetic genes (Fernandes et al., 1998). More than one AflR binding site is common in promoters of AF genes (Cary et al., 2009; Cary et al., 2000). Despite the differences between *A. nidulans* and *A. flavus* AflR, the function is conserved (Yu et al., 1996). Deletion *aflR* (Δ*aflR*) strain in *A. flavus* and *A. nidulans* fails to produce AF and ST respectively. Additionally, heterologously expressed *A. flavus aflR* in the deletion *A. nidulans* Δ*aflR* strain is able to activate the ST gene cluster, supporting functional conservation (Yu et al., 1996). In *A. parasiticus*, a microarray comparison of Δ*aflR* and wild-type strains revealed eighteen genes involved in AF pathway to be differentially expressed (Price et al., 2006). Constitutive expression of *aflR* was shown to increase the transcript of AF cluster genes along with 50-fold greater aflatoxin production (Flaherty and Payne 1997).

In addition to AflR, AflS also plays a role in the regulation of AF biosynthesis (Meyers et al., 1998; Chang et al., 1993; 2002; Chang, 2003). *aflS* lies next to *aflR* in the AF cluster. The gene products AflR and AflS were found to interact in *A. parasiticus* (Chang, 2003). Deletion of *aflS* has been shown to decrease expression of several biosynthetic genes in the cluster (Meyers et al., 1998), but does not alter *aflR* expression (Chang, 2003). The Δ*aflS* mutant still produces reduced but detectable levels of AF in *A. parasiticus* (Meyers et al., 1998). Interestingly, overexpression of *aflS*, particularly in combination with overexpression of *aflR* synergically increased expression of biosynthetic genes and AF production (Chang, 2003). *Aspergillus sojae*, is unable to produce AF in spite possessing the AF gene cluster in its genome; its *aflR* gene has a mutation that results in a truncated protein, which fails to interact with AflS (Chang, 2004).

Our genomic sequence analysis indicates that *aflS* is also present in *A. nidulans*, and that it is located next to *aflR* in the ST gene cluster (Calvo et al., unpublished data). As in the case of *aflS* in the AF cluster, *A. nidulans aflS* transcriptional direction is opposite with respect to that of *aflR*, sharing an intergenic region of 599 bp.

4. Global regulation

4.1 VeA and VeA-interacting proteins

Global regulatory mechanisms that control different cell functions, including morphogenesis and secondary metabolism tend to be conserved across fungal species. The *velvet* gene or *veA* is an example of global regulation. VeA is unique to fungi and is highly conserved in Ascomycetes (Myung et al., 2011). This regulator controls the morphological balance between sexual and asexual development (Kim et al., 2002; Li et al., 2006; Calvo, 2008). Interestingly, our laboratory showed for the first time that VeA also controls the production of numerous secondary metabolites, including ST and AF production in *A. nidulans,* and *A. flavus* and *A. parasiticus* (Kato et al., 2003; Calvo et al., 2004; Duran et al., 2007; Carry et al., 2007). In *A. nidulans* and later in *A. flavus* it has been demonstrated that

veA is necessary for *aflR* transcription, and therefore controls the expression of the ST/AF clustered genes (Kato et al., 2003; Duran et al., 2007).
The effect of VeA on secondary metabolism is broad. In *A. nidulans* the production of other metabolites, including penicillin, is also affected by the absence of *veA* (Kato et al., 2003). In *A. flavus*, the biosynthesis of other mycotoxins is also *veA*-dependent, such as the case of cyclopiazonic acid and aflatrem, where we showed that expression of aflatrem genes requires a *veA* wild-type allele (Duran et al., 2007).
VeA is also conserved across fungal genera, for example the *veA* homolog FvVE1 in *Fusarium verticilliodies* (Li et al., 2006; Myung et al., 2009), *AcveA* in *Acremonium chrysogenum* (Dreyer et al., 2007) and *ve-1* in *Neurospora crassa* (Bayram et al., 2008). Studies in these organisms also link *veA* to secondary metabolism. The *veA* regulatory system has been extensively reviewed (Calvo, 2008). VeA is transported to the nucleus (Stinnett et al., 2007) where it forms interactions with other regulatory proteins that also influence morphogenesis, and secondary metabolism, including production of mycotoxin (Purschwitz et al., 2008; Calvo, 2008; Bayram et al., 2008; Bayram et al., 2010).
In *A. nidulans* it has been shown that VeA interacts with a protein called LaeA (Bayram et al., 2008). *laeA* is also necessary for ST/AF production. Unlike *veA*, which has been shown to control *A. nidulans* asexual/sexual morphological development, *laeA* has a mild effect on morphogenesis, influencing Hülle cell numbers (Bayram et al., 2010). However, in *A. flavus* both *veA* and *laeA* have an important role in morphogenesis, being necessary for sclerotial production (Duran et al., 2007; Duran et al., 2009; Kale et al., 2008). LaeA is a putative methyl transferase, with an S-adenosylmethionine binding domain (Bok and Keller, 2004). In *A. flavus* it has been shown that *laeA* is a negative regulator of *veA* transcription (Kale et al., 2008). In addition, increased expression of *veA* (in a strain with multiple *veA* copies) results in decreased *laeA* expression (Amaike and Keller, 2009), suggesting a mutual negative transcriptional regulatory feedback control.
In addition to transcriptional regulation between *veA* and *laeA*, recent studies also suggest posttranslational regulation (Bayram et al., 2010); a new form of the VeA protein with higher molecular weight has been detected in Δ*laeA* strains, indicating that in the wild type, *laeA* prevents a modification of the VeA protein (Bayram et al., 2010).
Another protein from the *velvet* family, VelB, has been shown to interact with VeA. The Δ*velB* strain produces a reduced and delayed but still detectable amount of ST. VelB protein also interacts with VosA, a positive regulator of sporogenesis (Ni and Yu 2007; Bayram et al., 2010). Homologs of *velB* and *vosA* are present in *A. flavus* genome (data not shown). Other VeA-interacting proteins, such as the light-response mediating proteins FphA, LreA and LreB (Purschwitz et al., 2008), also influence morphological and metabolic changes in response to environmental stimuli affecting mycotoxin production (see section below corresponding to the effect of light).

4.2 G protein signalling

Several cellular functions, including fungal growth, morphogenesis and secondary metabolism are governed by G-protein signalling pathways. For example, the *A. nidulans fadA* gene encodes an α subunit of an heterotrimeric G protein complex where FadA interacts with Gβ (SfaD) and Gγ (GpgA) subunits (Rosen et al., 1999; Seo et al., 2005). Mutations in FadA blocking the intrinsic GTPase activity results in a permanently active stage of this protein (Hicks et at., 1997). It is likely that activation of FadA upregulates

adenylyl cyclase and cAMP-dependent kinase PKA. Activation of FadA or overexpression of PKA results in an increase in vegetative growth and reduction of asexual development,as well as reduction in ST production (Hicks et al., 1997; Shimizu and Keller, 2000). It has been shown that overexpression of PKA represses *aflR* expression (Shimizu and Keller, 2000). In addition to transcriptional regulation of ST genes, PKA also regulates ST production at posttranslational level. PKA negatively regulates the localization of AflR protein in the nucleus by phosphorylation (Shimizu et al., 2003). In *A. parasiticus* PKA also negatively regulates AF production (Roze et al., 2004). Additionally, expression of the FadA constitutive active form also resulted in a decrease of AF intermediates and a reduction in conidiation in *A. parasiticus* (Hicks et al., 1997). These findings strongly indicate a conservation of the FadA-PKA signaling pathway in regulating ST and AF production.

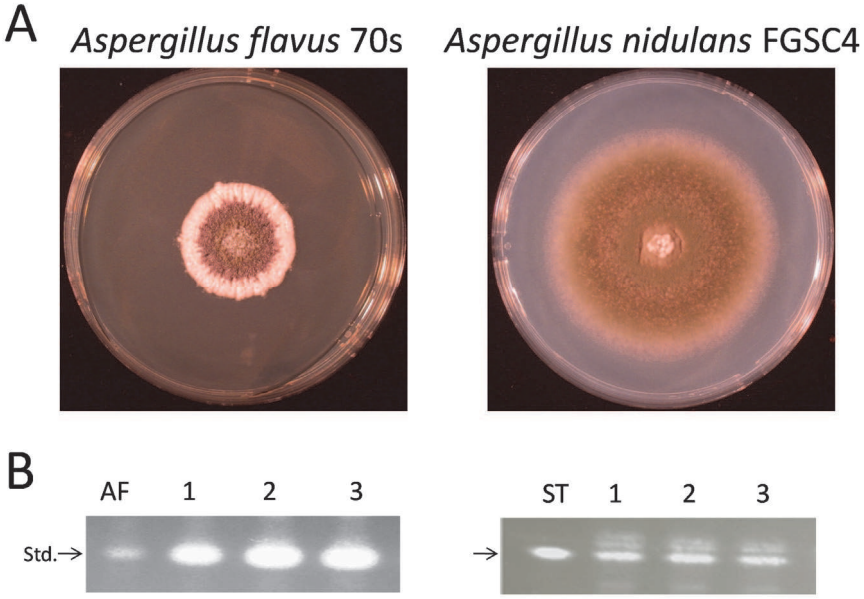

Fig. 2. *Aspergillus flavus* (70S) and *Aspergillus nidulans* (FGSC 4) wild type strains. A) Point-inoculated cultures incubated for five days. B) Thin layer chromatography analysis of the mycotoxins aflatoxin and sterigmatocystin. The chemical analysis was carried out as previously described (Kato et al., 2003; Duran et al., 2007). Std. = Standard, AF = Aflatoxin, ST = Sterigmatocystin. 1-3 represent replicates.

In the wild type, FadA is regulated by FluG, which activates transcription of *flbA*, encoding a GTPase activating protein that negatively regulates FadA (Lee and Adams, 1994; 1996; Seo et al., 2003. Both, *fluG* and *flbA* are necessary for ST production (Calvo et al., 2002). Homologs are also present in the *A. flavus* genome (data not shown).
Further studies showed of role of the FadA-interacting proteins, SfaD and GpgA subunits, and PhnA, a positive regulator of Gβγ, as positive regulators of *aflR* and consequently ST biosynthesis (Seo and Yu, 2006).

4.3 MpkB signalling

In eukaryotes **M**itogen **A**ctivated **P**rotein (MAP) kinases play a crucial role in signal transduction to the nucleus to control differentiation and cell growth (Banuett, 1998; Bradwell, 2006; Brown et al., 1996). For instance, in *Saccharomyces cerevisiae*, the MAP kinase *FUS3* is known to regulate mating (Doi et al., 1994; Zhan et al., 1997). In *A. nidulans* and other filamentous fungi, homologs of *FUS3* have been characterized (i.e. Atoui et al., 2008; Chen et al., 2004; Cousin et al., 2006; Di Petro et al., 2001; Jenczmionka et al., 2003; Li D. et al., 2005; Mey et al., 2002; Moriwaki et al., 2007; Rauyaree et al., 2005; Ruiz-Roldman et al., 2001; Takano et al., 2000; Xu et al., 1996;). Our laboratory has shown that the *A. nidulans FUS3* homolog, *mpkB*, controls sexual development and secondary metabolism (Paoleti et al., 2007; Atoui et al., 2008). *mpkB* affects expression of *laeA*, a global regulator of secondary metabolism (See Section Global Regulation). The *A. nidulans* Δ*mpkB* strain presents decreased transcript levels of *aflR* and of ST structural genes, consequently the Δ*mpkB* mutant failed to produce ST. Production of other secondary metabolites such as the antibiotic penicillin and the anti-tumoral compound terrequinone A was also affected (Atoui et al., 2008). Since *FUS3* homologs are also present in other fungal species, its role may be conserved in the AF-producing fungi.

4.4 Metabolic channeling

Chanda et al. (2009) also showed the importance of aflatoxigenic vesicles called aflatoxisomes in aflatoxin biosynthesis, storage and transport in *A. parasiticus*. High vesicle numbers were directly linked to AF-inducing growth conditions and inversely linked to lower expression of *vb1* (homolog of *AvaA* in *A. nidulans*) and *vps16* (another protein of Class C Vps tethering complex blocked by Sortin3). The wild type strain of *A. parasiticus* showed decreased transcript levels of *vb1* and *vps16*, whereas transcripts levels were constant in the Δ*veA* strain. This suggests a direct role for VeA in aflatoxisome production by down-regulating *vb1* and *vps16*.

4.5 Chromosome remodeling

Secondary metabolism gene clusters are often located in the chromosomal near-telomeric regions (Nierman et al., 2005; Rehmeyer et al., 2006), associated with epigenetic regulation. In 2004, Roze et al. identified CRE1 protein binding sites involved in the cAMP response. CRE1 protein recruits a histone acetyl transferase to the promoters of AF genes, resulting in acetylation of histones, specifically H4, favoring transcriptional activation (Shahbazian and Grunstein, 2007; Spencer and Davie, 1999; Daniel et al., 1998). Roze et al., (2007) showed the effect of histone H4 acetylation on transcript level of "early", "middle" and "late" genes of the AF cluster that leads to aflatoxin in *A. parasiticus*.The proposed model involves the activation of cAMP/PKA pathway by a decrease of glucose sensed by G-protein couple receptors. Activation of PKA would lead to CRE1 protein binding and recruiting of the histone acetyl transferase. This is followed by histone acetylation and AF gene expression, enhanced by AflR binding to the accessible promoter regions.

The effect of histone acetylation on mycotoxin production was further supported by *A. nidulans* studies, where deletion of the histone deacetylase gene, *hdaA,* lead to an increase in ST production by activation of transcription of ST genes. Deletion of *hdhA* also increased the production of other secondary metabolites such as penicillin (Shwab et al., 2007).

5. Environmental factors

5.1 Light

The effect of light on production of AF in *A. flavus* and *A. parasiticus* has been previously described (Joffe and Lisker, 1969; Bennett and Dunn et al., 1981). In these studies light-mediated regulation of mycotoxin production was conditioned by the incubation temperature. Light regulates morphological development as well as ST production in *A. nidulans*. A strain with a *veA* wild-type allele (*veA*+) develops asexually forming conidiophores in the light, while in the dark this fungus preferentially forms fruiting bodies called cleistothecia. *A. nidulans* produces more ST in the dark than in the light when cultured on glucose minimum medium. In the dark VeA is efficiently transported to the nucleus by the importin-α KapA (Stinnett et al., 2007), leading to ST biosynthesis. Interestingly, *A. nidulans veA1* mutants, with a truncated VeA protein missing the first 36 amino acids, produce less toxin compared to the *veA*+ strains (Stinnett et al., 2007). The truncation at the VeA N-terminal in *veA1* strains results in alterations of the binding with KapA and VeA transport (Stinnett et al., 2007; Araujo-Bazan et al., 2009).

Differential VeA localization was observed using blue and red light. Blue light had a similar effect to that with white light, decreasing VeA transport to nucleus, however, efficient nuclear localization was observed under red light, a result similar to that in dark conditions (Stinnett et al., 2007). This coincides with greater ST production under red light as compared to blue light. A red phytochrome-like protein, FphA, interacts directly with VeA in the nucleus (Purschwitz et al., 2008). Also, LreA and LreB, orthologs of the *N. crassa* blue collar protein CW1 and CW2 respectively, interact with VeA indirectly via FphA. Under light FphA negatively regulates VeA nuclear transport (Purschwitz et al., 2008). FphA and LreA/B proteins have opposite effects regulating secondary metabolism. Δ*fphA* strain of *A. nidulans* produced more ST than the wild type, whereas a reduction of toxin is observed in the Δ*lreA* and Δ*lreB* mutants. Homologs of *fphA*, *lreB* and *lreA* are present in the *A. flavus* genome, suggesting that the corresponding gene products could also be involved in AF regulation (data not shown).

5.2 Carbon source

It has been shown that AF is produced from glucose derived acetyl-CoA (Buchanan and Lewis, 1984, Shantha and Murthy, 1981). Simple sugar like glucose, sucrose, fructose or sorbitol enhances AF production, while complex carbon sources like peptone, galactose, xylulose, mannitol and lactose are not conducive to AF biosynthesis (Calvo et al., 2002 and references therein). A microarray analysis conducted on *A. parasiticus* showed that AF genes were differentially expressed when comparing expression levels in YE (medium with low sugar) and YES (medium with sucrose) (Wilkinson et al., 2007). A 10-fold increase in AF was observed within 48 hours in YES medium.

Interestingly, in a recent *A. nidulans* study we showed that the concentration of glucose is predominant to the effect of light on ST production (Atoui et al., 2010). Higher levels of ST were produced in the dark at low glucose concentration (1%) with respect to those produced in light cultures, however, more ST was observed in cultures exposed to light when higher amounts of glucose were added (i.e. 2%). These results also correlate with differential *aflR* expression levels in each case (Atoui et al., 2010). These findings indicate that the response to different environmental factors, such as light and carbon source on secondary metabolism occur integratively leading to an adaptation to a complex environment.

5.3 Nitrogen source

Nitrogen metabolism affects mycotoxin production. However the effect of nitrogen varies depending on the nitrogen source utilized. Whereas organic nitrogen sources induce AF production, nitrate as the sole nitrogen source in the medium is non-conducive to AF production (Georgianna and Payne, 2009). This differs from ST production in *A. nidulans*, where ST levels increase in medium with nitrate and are reduced when ammonia is used (Feng and Leonard, 1998).

In *A. parasiticus*, Chang et al.(2000) reported a major nitrogen utilization regulatory factor called AreA. This protein has been previously well characterized in *A. nidulans* where it has been shown to be a member of the GATA family of transcription factors and mediates nitrogen metabolite repression (Wilson and Arst, 1998). Overexpression of *aflR* in *A. parasiticus* overcomes the nitrate inhibition of AF production suggesting that AreA regulatory effect on mycotoxin biosynthesis could be linked to *aflR* regulation (Chang et al., 1995). Later it was demonstrated that AreA is able to bind to the intergenic region between *aflR* and *aflJ*, where several GATA binding sites are located (Chang et al., 2000).

Analysis of the effect of nitrate on AF biosynthesis and *aflR* and *alfS* expression in different *A. flavus* strains showed variability. This could be due variation in the number of AreA binding sites present in the intergenic region between *aflR-aflJ* (Ehrlich and Cotty, 2002). In addition, Marzluf (1997) suggests that nitrate utilization requiring induction and expression of several enzymes for reduction of nitrate to ammonium, could cause a delay in nitrogen utilization for AF biosynthesis. This may also be viewed as inhibition of biosynthesis (Ehrlich and Cotty, 2002).

5.4 Temperature

The effect of temperature on ST and AF biosynthesis is not conserved. While AF is produced between 25-30°C, ST is produced at a higher temperature, 37°C. Surprisingly, Schmidt-Heydt et al. (2009) showed that *aflS* (*aflJ*) expression levels were high at 37°C, however only low amounts of AF were detected. In addition, a microarray analysis performed by OBrian et al. (2007), showed 144 genes to be differentially expressed when *A. flavus* was grown at 28°C compared to cultures at 37°C. Out of these, 103 genes were upregulated at 28°C, 25% of which are involved in secondary metabolism. In this case, not only *aflJ*(*aflS*) but also *aflR* expression levels were similar at both 28°C and 37°C.

A SILAC study by Georgianna et al. (2008) on *A. flavus* revealed differences in protein levels at 28°C and at 37°C, while RNA transcripts did not change. This could be attributed to posttranslational regulation, where enzymes might not be present or as functional at the higher temperatures. This could also be attributed to the fact that in this study the authors only analyzed the cytosolic proteins, therefore concentration levels might vary due to different subcellular location or possible membrane interactions. Liu and Chu (1998) reported differences in AflR protein levels at 29°C when compared to those at 37°C using anti-AflR in *A. parasiticus*. AflR levels were 4 times lower at 37°C when compared to protein levels at 28°C. The difference in temperature-dependent AF production is most likely not related to phosphorylation of AflR by PKA, since the AflR subcellular localization does not change at 28°C or 37°C (Georgiana and Payne, 2009).

5.5 pH

It has been reported that *Aspergillus* species produce more AF/ST at acidic pH as compared to alkaline pH (Keller et al., 1997), which in *A. nidulans* correlates with an increase in *stcU*

expression in acidic environment. This may be attributed in part to the role of alkaline transcription factor PacC, a zinc finger transcription factor that binds to the consensus sequence 5'-GCCAAG-3' (Tilburn et al., 1995). Under acidic condition PacC is in its inactive form whereas under alkaline conditions, PacC is proteolytically cleaved to gain its active form. In its active form PacC activates expression of alkali expressed genes and represses acidic expressed genes by binding to the consensus sequence in the promoter region (Tilburn et al., 1995). PacC binding sites have been reported in the promoter of *aflR*, and putative binding sites have also been found on various genes in the cluster of ST/AF (Ehrlich et al., 1999; Keller et al., 1997). Putative binding sites have also been found in the promoter region of *veA* in different *Aspergilli* (Calvo et al., unpublished data), which, as mentioned above, is necessary for ST/AF production (Kato et al., 2003; Calvo et al., 2004; Duran et al., 2007).

Interestingly, PacC has antagonistic roles in controlling penicillin and mycotoxin production (Espeso et al., 1993). PacC-dependent penicillin production and expression of *ipnA*, gene encoding the isopenicillin N synthetase, increased under alkaline conditions when compared to acidic conditions.

In contrast with the reports mentioned above, a recent study by Delgado-Virgen and Guzman-de-Pena (2009) showed increased ST production in alkaline pH with respect to the levels produced at acidic pH. According to this report, *aflR* transcript levels are elevated in the "alkalinity mimicking" pacCc14 mutant. This opposite effect with respect to previous reports could be due to differences in culture conditions utilized in these two studies (for example Keller et al. used complete minimal medium while Delgado-Virgen and Guzman-de-Pena used Käfer minimal medium).

5.6 Plant metabolites

Fungal morphogenesis and secondary metabolism can be affected by plant-based compounds (Holmes et al., 2008). For example, volatile aldehydes, jasmonic acid and methyl jasmonate from the plant lipoxygenase pathway have been shown to reduce AF production (i.e. Zeringue and McCormick, 1990, Wright et al., 2000; Norton, 1999; Bhatnagar and MaCormick, 1988; Goodrich-Tanrikulu et al., 1995). Precursors of the lipoxygenase pathway called oxylipins, a family of oxidized polyenoic fatty acids, are found in different kingdoms, including plantae, fungi and monera (Tsitsgiannis and Keller, 2006). They might contribute to a communication established across kingdoms (Tsitsgiannis et al., 2004). In *A. nidulans,* oxylipin signal molecules called psi factors (Champe et al., 1987), are similar to 13S-hydroperoxy linoleic acid and 9S-hydroperoxy linolenic acid commonly found in plants (Calvo et al., 1999; Calvo et al., 2001; Tsitsigiannis et al., 2004). Both 9S-hydroperoxy linoleic acid and 13-hydroperoxy linolenic acid have been shown to differentially affect mycotoxin production; while the former has a stimulatory effect, the latter repressed its synthesis (Burow et al., 1997). Also, these fatty acids affect the balance between sclerotia/cleistothecial formation and conidiation in *A. flavus*, *A. parasiticus* and *A. nidulans* (Calvo et al., 1999). It is possible that these compounds of plant origin could mimic or interfere with the fungal psi factor regulatory mechanism. In *A. nidulans*, several oxylipin genes, *ppoA*, *ppoB* and *ppoC,* are responsible for synthesis of linoleic- and linolenic-derived psi factors. ST analysis of oxylipin deletion strains reveal that Δ*ppoB* and Δ*ppoC* strains produced more toxin than the control strain. On the other hand, Δ*ppoA* strain produced less toxin than the wild type. Δ*ppoA*, Δ*ppoC* and Δ*ppoA*, Δ*ppoB*, Δ*ppoC* double and triple mutants trains did not produce any detectable ST (Tsitsgiannis and Keller, 2006). The findings described indicate a direct correlation between presence of oxylipins and mycotoxin production.

Other compounds such as ethylene and CO_2 have been described to reduce AF production (Cary et al., 2009, and reference herein), while other molecules such as 2-ethyl-1-hexanol stimulate mycotoxin biosynthesis or affect it in a dose-dependent manner, such as the case of 2-buten-1-ol (Roze et al., 2007). Interestingly, ethylene, 2-ethyl-1-hexanol and 2-ethyl-1-hexanol have also been found to be produced by *Aspergillus* species, such as *A. parasiticus* and *A. nidulans,*where they could play a role as signal molecules (Roze et al., 2004; Roze et al., 2007).

5.7 Nitric oxide

Other gaseous molecules have been described as signaling molecules in higher eukaryotes. Plamer et al. (1987) and Ignarro et al. (1987) showed that the endothelium-dependent relaxing factor (EDRF) was indeed nitric oxide (NO), produced from L-Arginine (Palmer et al., 1988). Hausladen and Gow et al. (1998) identified a flabohemoglobin 'denitrosolase' in *E. coli* able to metabolize NO to nitrate. Thus flabohemoglobins were showed to decrease nitrosative stress by detoxifying NO. At low levels, NO is an important signaling molecule controlling numerous cell functions in higher eukaryotes (Wendehenne et al., 2001, Romanov et al., 2008; Roselli et al., 1998; Masuda et al., 2001; Kim et al., 2007; Lamattina et al., 2003). However,until recently there were no studies of NO as a signaling molecule in fungi. In *A. nidulans* two flabohemoglobins, FhbA and FhbB, have been reported (Schinko et al., 2010). A recent study in our laboratory (Baidya et al., 2011) showed a link between NO and morphological development and ST production in *A. nidulans*. This was the first report of the effect of NO in morphogenesis and secondary metabolism in the fungal kingdom (Baidya et al., 2011). Deletion of *fhbA* results in an increase of sexual development and decrease of ST biosynthesis (Baidya et al., 2011). The reduction of ST was linked to a decrease of *aflR* expression. Homologs of *fhbA* and *fhbB* are present in the *A. flavus* genome (unpublished data). The implications of this study suggest a broader possible regulatory effect of NO that might also impact AF production as well as the production of other fungal metabolites.

6. Conclusion

AF, commonly produced by *A. flavus* and *A. parasiticus*, is a potent mutagenic and carcinogenic natural compound that constitutes a health threat worldwide. To set the basis for the development of efficient control strategies to prevent or reduce the negative impact associated with AF contamination it is essential to uncover the regulatory networks that lead to mycotoxin biosynthesis in these fungi. In this review we have shown that studies in the model filamentous fungus *A. nidulans* have facilitated rapid progress in this field, revealing genetic mechanisms governing the production of the mycotoxin ST that are also found conserved in the regulation and production of AF by *A. flavus* or *A. parasiticus*. This, combined with the availability of new technologies leading to the genome sequencing of *A. nidulans, A. flavus* and other filamentous fungi, has further expanded our knowledge on AF/ST regulation and biosynthesis.

The elucidation of the genetic mechanisms that operate in response to different environmental factors is of particular importance, since some of these responses are adaptations that allow these fungi to colonize plants leading to AF contamination of crops. Part of these response mechanisms to environmental cues involves the participation of proteins and formation of protein interactions that are unique to fungi, for example VeA and

VeA-interacting proteins, initially characterized in *A. nidulans*. For these reasons these protein complexes constitute potential targets for control strategies to reduce the production of AF and possibly mycotoxin production in other fungi.

Although there are some disparities in the regulation of ST and AF, most of the control mechanisms governing the synthesis of these mycotoxins are conserved. *A. nidulans* is an excellent model organism to elucidate the complexity of these regulatory networks and provide insight in remediating the impact of AF contamination.

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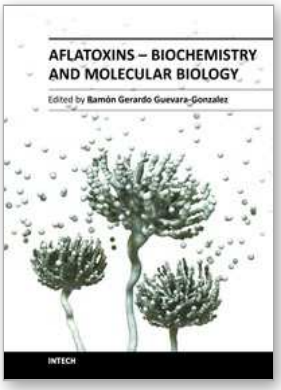

Aflatoxins - Biochemistry and Molecular Biology

Edited by Dr. Ramon G. Guevara-Gonzalez

ISBN 978-953-307-395-8
Hard cover, 468 pages
Publisher InTech
Published online 03, October, 2011
Published in print edition October, 2011

Aflatoxins â€“ Biochemistry and Molecular Biology is a book that has been thought to present the most significant advances in these disciplines focused on the knowledge of such toxins. All authors, who supported the excellent work showed in every chapter of this book, are placed at the frontier of knowledge on this subject, thus, this book will be obligated reference to issue upon its publication. Finally, this book has been published in an attempt to present a written forum for researchers and teachers interested in the subject, having a current picture in this field of research about these interesting and intriguing toxins.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Ana M. Calvo and Sourabh Dhingra (2011). Conserved Regulatory Mechanisms Controlling Aflatoxin and Sterigmatocystin Biosynthesis, Aflatoxins - Biochemistry and Molecular Biology, Dr. Ramon G. Guevara-Gonzalez (Ed.), ISBN: 978-953-307-395-8, InTech, Available from: http://www.intechopen.com/books/aflatoxins-biochemistry-and-molecular-biology/conserved-regulatory-mechanisms-controlling-aflatoxin-and-sterigmatocystin-biosynthesis

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